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(71) Applicant *(for all designated States except US):* CENTRE FOR PLANT BREEDING AND REPRODUCTION RESEARCH (CPRO-DLO) [NL/NL]; Droevendaalsesteeg 1, NL-6708 PB Wageningen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* VAN TUNEN, Arjen, J. [NL/NL]; Wim Sonneveldstraat 31, NL-6708 NA Wageningen (NL). MOLLEMA, Chris [NL/NL]; De Rauwendaal 21, NL-6666 BX Heteren (NL). ANGENENT, Gerco, C. [NL/NL]; Leo Gestelstraat 5, NL-6717 RH Ede (NL). DONS, Johannes, J., M. [NL/NL]; Nassauweg 30, NL-6703 CJ Wageningen (NL).

(74) Agent: DE HOOP, E.; Octrooibureau Vriesendorp & Gaade, P.O. Box 266, NL-2501 AW The Hague (NL).

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(57) Abstract

The present invention provides plants with altered flower morphology, characterized in that said plants have a recombinant polynucleotide integrated into their genome, essentially comprising of an inhibitory gene, which upon proper expression in the floral meristem cells is capable of inhibiting the development and determination of floral primordia into petal and/or stamen primordia. Said plants have a disturbed relation with pest insects which are attracted by the flowers. As a result of that said plants have an increased resistance against those pest insects and the primary but also the secondary damage caused by these insects is significantly reduced.

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A method for obtaining a plant having altered floral morphology and a method for protecting plants against pest insects.

FIELD OF THE INVENTION.

The invention is related to recombinant DNA, more in particular to recombinant DNA in relation to genetic manipulation of plants. The invention is further related to plants which have modified floral organs and enhanced resistance to insect plagues and to secondary fungal infections, due to expression of the said recombinant DNA, as well as part of the said plant which are either sexually or asexually reproducible, or both.

BACKGROUND AND STATE OF THE ART.

Protection of crops against insect plagues is one of the major goals of modern plant breeding. Nowadays for crop protection growers heavily depend on the use of chemical agents. However these chemical agents have some important disadvantages such as a substantial environmental pollution, the short time a chemical can be used due to the induction of insensitivity of the insects to the chemical and unwanted side effects on other non-harmful insect species. Therefore introduction of resistance genes via breeding programmes has become an increasingly important method of crop protection (for a review see for instance Dons et al., 1991). Recently the modern genetic modification techniques provide new ways to introduce isolated resistance genes into crops. Following such a scheme it, for instance, has been possible to introduce resistance against insects of the *Lepidoptera* and *Coleoptera* classes by the introduction of genes from the bacterium *Bacillus thuringiensis* (Feitelson et al., 1992). However, alternative methods are still highly

desirable because BT-resistance can be easily broken by the target organisms and a number of important insect classes are not sensitive to BT genes.

In summary it can be stated that there is a strong commercial interest in the introduction of reliable insect resistances in crop species which have to be highly disadvantageous to the pest insect but on the other hand harmless to other insect species or animals including humans. The present invention provides a method which has such advantages. It involves the modification of floral morphology of the plant.

With respect to molecular processes involved in the formation of floral organs during the last three years a rapidly increasing amount of knowledge has been generated, especially for two plant species: *Arabidopsis thaliana* and *Antirrhinum majus*. Several recent studies have been focussing on the development of flowers (for reviews: Gasser, 1991; Coen, 1991). A rapidly increasing number of genes involved in floral organogenesis has been isolated from these plant species and characterized at the molecular level. The flower homeotic gene *agamous* (*ag*) and the *agamous-like* (*agl*) gene family have been cloned from Arabidopsis (Yanofski et al., 1990; Ma et al., 1991). Two homeotic genes, *deficiens* (*defA*) and *floricaula* (*flo*) have been isolated from Snapdragon (Sommer et al., 1990; Coen et al., 1990). Recently, Pnueli et al. (1991) reported the isolation and characterization of a homeotic gene family expressed in tomato flowers. Sequence analysis has revealed that most of the proteins encoded by these homeotic genes contain a region with a striking homology to the putative DNA binding domain of transcription factors from humans (SRF; Norman et al., 1988) and Yeast (MCM1; Passmore et al., 1988). This conserved motif was designated as the "MADS box" (Schwarz-Sommer et al., 1990).

A number of these MADS box genes are only expressed during floral development and serve important functions with respect to the development of floral organs. For instance the *agamous* and *deficiens* genes play important roles in the development of petals and stamens. Natural mutants for these genes possess flowers without petals and stamens and instead extra rows of sepals and carpels are formed.

DEFINITIONS.

Antisense gene: a gene, or a nucleotide sequence derived thereof, having a homology of more than 50%, preferably more than 80% with a target gene as defined herein and which is linked to a promoter in the inverse 5' to 3' orientation with respect to the target gene.

Apomixis: a form of asexual reproduction in which seed is produced but the embryo develops from an unreduced cell of the ovule without fusion of male and female gametes.

Gene: a nucleotide sequence that can be expressed in the form of an RNA molecule and/or a polypeptide.

Inhibitory gene: a gene or antisense gene, expression of which ultimately leads to inhibition of expression of a target gene as defined herein.

MADS box gene: a gene having a region of about 55 amino acids with more than 30% , preferably more than 50%, amino acid homology with the yeast *MCM1* gene, with the *Arabidopsis Agamous* gene, the *Antirrhinum Deficiens* gene and/or the human *SRF* gene.

Parthenocarpy: the phenomenon that (seedless) fruits are formed without a fertilization event.

Promoter: a nucleotide sequence which is capable of promoting expression of a gene or antisense gene, or nucleotide sequences derived thereof, said expression being in the form of an RNA molecule and/or polypeptide.

Sense/co-suppression gene: a gene, or a nucleotide sequence derived thereof, having a homology of more than 50%, preferably linked to a promoter in the normal 5' to 3' orientation with respect to the target gene.

Target gene: a gene, expression of which is to be inhibited by proper expression of a suitable inhibitory gene as herein defined.

SUMMARY OF THE INVENTION.

The present inventors have found that it is possible to disturb insect/flower relations in such a way that the pest insects are not attracted by the crop plants as a result of genetically modified target flowers. More specifically, the present inventors have found a method for protecting plants against pest insects by altering the flower morphology of the plant such that the petals and/or stamens are completely or partially removed. The modification of the flower morphology of the plant is suitably and preferably carried out by introducing a specific recombinant polynucleotide in the genome of the plant.

It has been found that the genetically modified plants according to the invention possess an increased resistance against pest insects attracted by flowers or insects feeding on the pollen. Moreover, it has been found that said genetically modified plants are less sensitive to organisms such as fungi which cause secondary infections at lesion sites caused by insect predators.

The present invention provides recombinant polynucleotides which can be suitably used for obtaining a plant having the petals and/or stamens completely or partially removed, essentially comprising:

(a) an inhibitory gene either being a gene capable of inhibiting

expression of a target gene in the said plant encoding a MADS box protein involved in flower organogenesis developmental programmes or being a toxin gene causing cell death, and

(b) a promoter that is active in the petals and/or stamens of said plant, operably linked to said inhibitory genes as to achieve expression thereof in floral organ primordia of said plant.

A preferred target gene according to the invention encodes MADS box proteins specifying the determination of floral primordia into petal and/or stamen primordia. Especially preferred target genes encode the *fbp1* or *fbp2* proteins as disclosed hereinafter or proteins which are homologous therewith. A preferred toxin causing cell death encodes products toxic for plant cells.

Especially preferred as toxin gene is a gene encoding an RNA degrading enzyme (RNAse). Such ribonucleases can be used for a depletion of specific cell types as was for instance shown in the case of tapetal cell from tobacco anthers by Mariani et al. (1990).

In a preferred embodiment of the invention the inhibitory gene is an antisense or sense/co-suppression gene directed against a MADS box target gene.

In another preferred embodiment according to the invention the promoter that is active in the floral primordia that give rise to the formation of petal and/or stamen organs comprises a highly active CaMV 35S promoter. In another embodiment the highly active promoter comprises a MADS box gene promoter. Preferred MADS box gene promoters are of the B-type e.g. the *deficiens* and the *globosa* promoters of *Antirrhinum*, and the *apetala 3* and the *pistillata* promoters of Arabidopsis. In a particularly preferred embodiment the high level promoter comprises the *fbp1* promoter from *Petunia*.

The invention also provides a method for obtaining a plant with modified floral organs, comprising the steps of

(a) transferring a recombinant polynucleotide as defined above to cells of a wild type plant with normal flowers,

(b) generating whole new plants from cells having incorporated said recombinant polynucleotide, and.

(c) selecting a plant that has flowers with the petals and/ or stamens completely or partially removed.

The invention provides in another aspect a recombinant plant genome, comprising incorporated therein a recombinant polynucleotide as defined above.

The present invention is especially useful for plant species for which in the course of crop production fully developed petals and/or stamens are not necessary. Preferred plant species according to the invention are parthenocarpic and/or apomictic plants. Highly preferred plant species according to the invention are *Cucurbita pepo*, *Pyrus*

communis, *Vitis vinifera*, *Solanum melongena*, *Carum carvi* and *Lycopersicum esculentum*. The most preferred plant species is cucumber (*Cucumis sativis*).

The invention further encompasses a cell, organ, fruit, seed or progeny derived from a plant having altered flower morphology as defined above.

The present invention provides plants without or with reduced petals and/or stamen organs and as a result of that have a disturbed relation with pest insects which are attracted by and feeding on the flowers. A thrips population developed slower on cucumber plants with modified flowers and the amount of leaf damage was reduced. In addition the genetic modification of floral organogenesis in which petals and/or anthers were completely or partly removed resulted in the formation of flowers which are more accessable for insecticides. Taken together said plants have an increased resistance against those pest insects and the primary but also the secondary damage is significantly reduced. The present invention thus provides an environmentally attractive and alternative method for obtaining plants with enhanced resistance against pest insects thereby also reducing the need for extensive and highly polluting chemical spraying.

The present invention can also be used to produce plants without or reduced petals thereby decreasing the size of the often massive flower screens. This has the advantage that the amount of photosynthesis increases dramatically as was reported recently by Rao et al. (1991) for a natural apetalous oilseed rape variety. Furthermore the present invention can also be used to produce plants without or with reduced stamens. The resulting absence of pollen will enhance resistance of strawberries against fungi as was reported by Simpson (1992) for natural strawberry mutants and the fungus *Botrytis*.

The present invention also provides plants with stamens transformed into carpels. As a result of that, genetically modified plants are produced with flowers which contain besides their normal whorl 4 gynoecium also an extra whorl 3 gynoecium. This has the advantage that extra seeds are produced and that the amount of seedset on one plant can be elevated. This part of the invention is especially usefull for plant species used for seed production. Preferred plant species according to the invention are belonging to the group of oilseed crops like safflower, sunflower, flax, lineseed, sesame and groundnut, the group of grain legume crops like amaranth, chenopod, oat, millet, barley, rice, rye, sorghum, triticale, wheat, maize, pigeon pea, chick pea, soybean, lentil, alfalfa, bean, pea, broad bean, cowpea and buckwheat and the group of stimulating beverages like coffee, cola and cacao. A highly preferred plant species according to the invention is rape seed (*Brassica napens*).

DESCRIPTION OF THE FIGURES.

Figure 1 Nucleotide and deduced amino acid sequence of the *fbp1* (A) and *fbp2* cDNA (B) used for reverse genetic techniques. The conserved amino acids used for the degenerated PCR primers are underlined. The intron/exon junctions are denoted by arrowheads. The *fbp1* cDNA sequences present in primers 1 and 2 used for amplification of the coding region by PCR are indicated by lines.

Figure 2 Diagrammatic representation of the *fbp1* gene (A), the chimeric GUS construct (B), the chimeric *fbp1* sense/co-suppression construct (C) and the *fbp2* sense/co-suppression construct (D).

Figure 3 Fbp1 driven GUS expression in eight transgenic *Petunia* plants (T5001, T5002, T5003, T5005, T5006, T5008, T5010, and T5011) containing pFBP12E. GUS activity was only detected in petals and stamens and not in (for instance) leaves.

Figure 4 Photograph showing different developmental stages of cucumber seedlings. The optimal stage for transformation is stage B (B).

Figure 5 Number of thrips larvae on leaves of flowerless plants (compartments A and C) or control plants (compartments B and D) one month after inoculation (ten weeks after sowing).

Figure 6 Damage to cucumber leaves of flowerless plants (compartments A and C) or control plants (compartments B and D) one month after inoculation (ten weeks after sowing).

Figure 7 Damage to cucumber leaves of flowerless plants (compartments A and C) or control plants (compartments B and D) three months after inoculation (eighteen weeks after sowing).

Figure 8 Mean fruit weight of cucumbers grown on flowerless plants from compartments A and C compared to control fruit from compartments B and D.

Figure 9 Phenotype of transgenic petunia plants with inhibited *fbp1* gene expression. The flowers have the 5 stamens transformed into 5 carpels which are fused to form an extra whorl 3 gynoecium.

Figure 10 Seedset on transgenic *fbp1* petunia plants with modified flowers (see also Figure 9). Extra seeds are produced by the whorl 3 gynoecium after pollination.

DETAILED DESCRIPTION OF THE INVENTION.

From the genome of the plant species *Petunia hybrida* a flower-specific gene entitled *fbp1* was isolated. This gene has features of a transcription factor and belongs to the group of MADS box genes. *Fbp1* mRNA was shown to be present only in whorl 2 and whorl 3 floral organs. It was also found that expression of a bacterial ß-glucuronidase (GUS) reporter gene in transgenic Petunia plants driven by the *fbp1* promoter was only detectable in whorl 2 and 3 organs. From the analysis of the same transgenic plants it could also be concluded that the *fbp1* promoter is activated during the differentiation stage of floral organ primordia. No expression was detected in other floral parts including the female reproductive organs, or in any of the vegetative plant parts (roots, stem, leaves).

Apparently this gene plays a crucial role in the determination of part of the floral meristem into petal and stamen primordia. Surprisingly experiments in which this gene was introduced into cucumber cells by particle gun bombardment showed that the *fbp1* promoter is also active in petals and stamen tissues of cucumber.

Transgenic petunia plants were generated in which the *fbp1* cDNA was overexpressed by the introduction of a gene construct consisting of the CaMV 35S promoter and the *fbp1* cDNA in the sense orientation. The transformation of the *fbp1* cDNA geneconstruct resulted in an inhibition of *fbp1* expression by the socalled cosuppression effect (Napoli et al., 1990; van der Krol et al., 1990). After the plants were allowed to flower, transgenic plants were identified in which surprisingly petal tissue was replaced by sepal tissue and on top of the anther pisteloid tissue was formed. This conversion leads to the formation of non-pigmented sepal-like petals and pisteloid anthers. Selfing of these primary transgenic plants resulted in a normal seedset. The offspring of these seeds could be divided into three different classes. The first class represented untransformed petunia W115 plants without the gene construct and with a normal W115 petunia phenotype. The second class represented a class of plants containing the transgenes in a heterozygote situation and showed a phenotype closely resembling the primary transgenic plants. The third class represented plants with the transgenes in a homozygote situation and a considerable more severe phenotype than the primary transformant. The transformation of petals into sepals was almost complete and the stamens were completely replaced by carpels. These carpels fused to each other and to the whorl 4 carpels. Surprisingly extra seedset in whorl 3 was observed when the modified flowers were pollinated.

The whorl 1 and 4 organs were not effected in both the heterozygote or in the homozygote transgenic plants. These homeotic conversions

observed reveal a close resemblance with the conversions observed in natural *Arabidopsis*, *Antirrhinum* and *Petunia* flower mutants in which petals are replaced by sepals and stamens are replaced by carpels. Thus it was concluded that by inhibition of *fbp1* mRNA synthesis the phenotype of *Petunia* whorl 2 and 3 floral organs could be altered without changing the whorl 4 pistel organs.

A second MADS box cDNA, designated *fbp2*, was isolated from a petal-specific cDNA library with the use of the MADS box of *fbp1* as a probe. RNA blot analysis revealed that the *fbp2* gene is expressed only in flowers and only in whorls 2, 3 and 4. Sense/co-suppression experiments in which a 35S CaMV-fbp2 construct was transformed to *Petunia* yielded transgenic plants with green corollas and petaloid anthers. From this it was concluded that using such a procedure it was again possible to generate transgenic plants with modified whorl 2 and 3 organs. It will also be possible to introduce similar geneconstructs into the cucumber genome. An experiment in this direction revealed that the cucumber accession IBPGR can be readily transformed provided that seedlings of the optimal developmental stage are used in the transformation procedure.

In another experiment the petals and stamens, but not the pistels, were removed daily by hand from cucumber flowers during a period of six weeks. At the start of the experiment 40 larvae of the Western Flower Thrips (*Frankliniella occidentalis*) were inoculated on each of the first two true leaves of 12 young cucumber plants. In this experiment it was surprisingly observed that the number of mature thrips insects and the number of thrips larvae on the leaves was significantly reduced (Figure 5). The reduced number of trips insects was accompanied by a high reduction of the damage of cucumber leaves (Figure 6 and 7). The removal of floral organs did not have effects on the growth and yield of the cucumber fruits because of the parthenocarpic nature of this crop (Figure 8). Thus it can be concluded that altering the flower morphology of cucumber plants by deleting the petals and stamens results in an increase of resistance to *Frankliniella occidentalis*.

EXPERIMENTAL.

DNA Methodology.

DNA isolation, subcloning, restriction analysis and sequencing were performed using standard procedures well known to persons skilled in the art (e.g. Maniatis et al., 1982). Isolation of DNA from individual *Petunia* transformants and DNA gel blot analysis were performed as described by Koes et al., 1987.

Polymerase Chain Reaction (PCR)

A Isolation of MADS Box Genes

Single stranded cDNA was synthesized by priming with the oligonucleotide 5'-CCGGATCCTCTAGAGCGGCCGC$(T)_{17}$-3' (prat 7) starting from 10 µg of total RNA isolated from young corolla tissue (according to Koes et al., 1989). A degenerated primer prat 8 with the sequence 5'-GGGGTACCAA(G/A)CGI(C/A)(G/A)I(A/C)(C/A)(T/C)GG(I/C)(T/C/A)T(I/C)(A/T)T(G/C/T)AA(G/A)AA(G/A)GC-3' based on the conserved MADS box sequence KRRNGLFKKA of the *DefA* gene from *Antirrhinum majus* was used for the initial PCR analysis. PCR analysis was performed in 100 µl of PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$) containing 80 and 100 pmol of 5' and 3' primer, respectively, and 200 µM of each deoxynucleotide triphosphate. Amplification involved 30 cycles with a denaturing time of 20s at 94°C, cooling down in 90 sec to 37°C, an anealing time of 30s, and an extension time of 6 min at 60°C. Amplified cDNA was fractionated on 1% agarose gel, revealing one clear fragment of about 0.8 kb. This band was isolated and subcloned into M13mp19 vector using BamH1 and KpnI restriction site present in the 5' and 3' primers, respectively. This cDNA insert (designated as *fbp1*) was used to screen the cDNA library and a complete cDNA clone of *fbp1* was isolated. This clone was used to rescreen the cDNA library under low stringency conditions (2xSSC, 60°C) and five additional MADS box cDNA clones were isolated including *fbp2*.

B Construction of Chimeric *Fbp1*-GUS Gene.

Prat 11, 5'-CCGGATCCCTCTCCCCATGGTTTCCCTTTCTC-3' and prat 14 5'-CGGGTCGACGTAAAACGACGGCCAGTGAATTG-3' were used to isolate an fbp1 promoter fragment by a PCR approach. The PCR was performed in 100 µl PCR buffer (see above) containing 100 pmol of prat 11 and 14. Amplification involved 30 cycles with a denaturing time of 20s at 94°C, an annealing at 55°C for 30s and an extension time for 2 min at 72°C. The resulting fragment of 240 bp was isolated from a normal 1% agarose gel and used for further subcloning.

Screening cDNA and Genomic Libraries.

A lambda gt11 cDNA library made from poly(A)$^+$ RNA of R27 corollas (van Tunen et al., 1988) was screened. Plating and screening of the library using *E. coli* Y1090 cells as host was performed according to the Promega protocol. Probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein, 1984), hybridization and washing of the Hybond-N membranes (Amersham) were performed at high stringency (65°C hybridization and final wash with 0.1 SSC; 1xSSC consists of 150mM NaCl, 15mM NaCitrate). The genomic library was made by insertion of partial

Sau3A fragments of R27 nuclear DNA into the vector lambda GEM12 (Promega). Approximately 150,000 plaques were sreened with ^{32}P-labelled *fbp1* cDNA, and positive clones were isolated and purified. Subsequently, the inserts or part of the inserts were subcloned into pEMBL vectors (Boehringer) and further analyzed by restriction enzyme analyses, hybridization and sequencing.

GUS Extractions and Fluorimetric and Histochemical GUS Assays.

Fresh material was collected from the transgenic plants and used for the GUS assays. GUS extractions were performed as described by Jefferson et al., (1987), by grinding the tissue with liquid N2. Fluorimetric GUS activity measurements were performed according to Jefferson et al., (1987). Protein concentrations were determined using the Biorad protein assay with Bovine serum albumin as a standard.

Histochemical localization of GUS activity was performed as described by Koes et al., (1990). Before staining the floral buds were cut into small slices with a razor blade. The X-Gluc staining was performed according to Jefferson et al., (1987) at pH 7.5 and in the presence of 0.5 mM K^+ ferricyanide/ferrocyanide. For analysis at the single cell level X-Gluc stained tissues were fixated and imbedded in Technovit 7100 according to the protocol of the manufacturer (Kulzer Histo-Tec). Using a microtome, 10 um thick sections were cut of which photographs were taken by bright and dark field microscopy.

Particle Gun Bombardment.

Mature flowerbuds were collected from cucumber plants and layered on a MS/agar plate. Bombardment was performed with a DuPont PDS1000 apparatus at 28.5 inch immediately after the floral tissues were collected. pFBP12E DNA (2.5 µg) was precipitated on 1.6 µm thungsten particles according to the method of van der Leede-Plegt et al. (1992). As a control 1.6 µm particles were used without adhering DNA. After bombardment the floral tissues were incubated for 2 days. Subsequently the floral tissue was stained for GUS enzyme activity according Jefferson et al. (1987).

Transformation of Petunia and Cucumber.

Petunia hybrida was transformed according to the method of Horsch et al., (1985). After shoot and root induction on kanamycin-containing media, plants were put in the soil and kept in a green house. Plants regenerated on kanamycin-less media) from leaf discs treated with the strain LBA4404 without a binary vector served as a control.

Regeneration of the cucumber variety IBPGR was done according to the method developed by Colijn-Hooymans et al. (1992). Cucumber seeds

were sterilized for 15' in 2% (w/v) NaOCl and washed three times with sterile water. The seeds were germinated in honey jars on sterilized (20' 120°C) MS medium supplemented with trypton L42 (Oxoid 500 mg/l), sucrose (30 g/l) and Imperial agar (6 g/l), pH5.6, at 25°C under cool white light (Philips FTD 36W/TL84, 16, 2500 lux). Cotyledons were excised from the seedlings at the developmental stage B and transversely cut into two parts. In developmental stage B the cotyledons are green and in a vertical position (Figure 4) which is in contrast to stage A were the cotyledons are still folded and are light green. The basal explant was inserted in induction medium with the basal wounded edge in contact with the medium. This induction medium consisted of MS salts and vitamins supplemented with sucrose (40 g/l), trypton L42 (500 g/l), 50 μM kinetin, 0.1 μM indoleacetic acid (IAA) and Imperial agar (6 g/l), pH 5.6, sterilized for 20' at 120°C. Petri dishes containing the explants were placed in a growth chamber at 25°C and 16 hrs of cool white light (Philips FTD 36W/TL 84, 750 lux). After 14 days on induction medium the explants were transferred to plant development medium which consists of MS salts and vitamins supplemented by trypton L42 (500 g/l), sucrose (20g/l), 0.5 μM kinetin, 0.1 μM IAA and Imperial agar (6 g/l). The medium was adjusted to pH 5.6 prior to autoclaving for 20' at 120°C.

As a bacterial strain for transformation *Agrobacterium tumefaciens* C58C1 containing a GUS-intron reporter gene (pMP90 + GUS intron; Van Canneyt et al., 1990) was used. The bacteria were grown for 16 hrs in 10 ml LB supplemented with kanamycin and rifampicin (50 mg/l) at 28°C in a shaking wather bath. After growth the bacteria were pelleted by centrifugation at 5000 rpm for 10'. The resulting pellet was resuspended in 10 ml liquid induction medium poured into petri dishes to be used for inoculation. Cotyledonary explants were placed for 2 minutes into the bacterial suspension. After this they were dried in sterilized paper discs and inserted in induction medium with the basal wounded edge in the medium. The inoculated discs were co-cultivated with Agrobacterium for 3 days at 25°C and 16hrs of 750 lux cool white light. Subsequently they were removed to the same medium supplemented with 250 mg/l cefotaxime, 200 mg/l vancomycin and 75 mg/l kanamycin. The cultures were placed at 25°C and 16 hrs of cool white light (Philips 36W/TL 84) at 750 lux. After 14 days the developed shoots were cut from the explants. Rooting of the developed shoots occured spontaneously on the same medium in honey jars. Young plants with two leaves were transferred to rockwool plugs and placed in the greenhouse. After this the plants were checked for GUS activity.

Growth of cucumber plants and removal of floral parts.

Female flowering, parthenocarpic cucumber plants (variety "Corona", De Ruiter Seeds) were used for insect resistance experiments. The plants were grown in pots containing peatsoil with osmocoat. The experiment was performed in four compartments of a greenhouse (designated A, B, C and D). Each compartment was 16 square meters in size and contained 16 plants. The compartments were strictly isolated from each other. The plants were grown at 25°C during day time (from 06.00 - 22.00) and 20°C during the night (from 22.00 - 06.00). Shoots were removed twice a week. Approximately two meters above the soil a wire was constructed which carried the top of the plant. From this top downwards two stems were maintained.

Starting five weeks after sowing all flowerbuds were removed daily from plants in compartments A and C wereas the control plants in compartments B and D were untreated. The flower buds were removed in such a way that the lower parts of the carpel remained intact. Six weeks after sowing 20 first instar and 20 second instar *Frankliniella occidentalis* larvae were introduced on the first two leaves of each plant in all four compartments. Eight weeks after sowing 200 mature female *Frankliniella occidentalis* insects were released in each compartment.

Ten weeks after sowing four leaves per compartment were cut at the same height from four random plants. The thrips larvae present on the leaves were counted and the lesion damage of the leaves was measured by image analysis according to Mollema et al., (1992). Eighteen weeks after sowing the damage of five leaves of four plants per compartment was determined again. Throughout the experiment fruits were harvested and the weight of the fruits was determined.

EXAMPLES.

I Isolation of MADS Box Genes Using a Polymerase Chain Reaction (PCR) Approach.

The isolation of floral morphology genes from Petunia was based on the use of a conserved sequence found in DNA binding domains of transcription factors of plant, fungal and human origin (MADS Box, Yanofski et al., 1990). A set of degenerated oligonucleotides deduced from the decapeptide sequence KRRNGLFKKA, 5'-GGGGTACCAA(G/A)CGI(C/A)(G/A)I(A/C)(C/A)(T/C)GG(I/C)(T/C/A)T (I/C)(A/T)T(G/C/T)AA(G/A)AA(G/A)GC-3', present within the MADS box was synthesized. These oligonucleotides and an oligo(dT) primer, 5'-CCGGATCCTCTAGAGCGGCCGC$(T)_{17}$-3', were used for an amplification of MADS box cDNA clones synthesized from floral mRNA by polymerase chain reaction

(PCR). Subsequently, the resulting PCR products were used to screen a lambda gt11-based petal-specific library (van Tunen et al., 1988). Initially, one cDNA clone, designated as *fbp1*, was isolated and analyzed in more detail. Rescreening of the library with *fbp1* as a probe resulted in the isolation additional MADS box cDNA clones including *fbp2*. The complete nucleotide sequences of *fbp1* (780 base pares) and *fbp2* (960 base pairs) were determined and are depicted in Figure 1. The sequence of the *fbp1* cDNA (Figure 1A) clone contains a large open reading frame with a calculated coding capacity of 24.6 kD as well as 5' and 3' non-coding regions. The N-termini of *fbp1* and *fbp2* contain the complete MADS box domain. Screening of a lambda ZAP based carpel-specific cDNA library made from RNA extracted from carpels isolated from young petunia W115 flowerbuds less than 1.0 cm in size with a mixed MADS box probe from the *fbp1* and *fbp2* cDNA under low stringency hybridization conditions yielded an additional eight cDNA clones.

The complete *fbp1* cDNA insert was used to isolate the *fbp1* gene from a genomic library of the Petunia line R27. Hybridization under stringent conditions revealed four positive clones, which were further purified. The nucleotide sequence of the *fbp1* gene in one of those clones as well as +/- 1 kb of upstream sequence were determined resulting in a genomic structure depicted in Figure 2A.

II Construction of Chimeric *Fbp1*-GUS Gene.

Using prat 11 and 14 a PCR experiment was performed on DNA from deletion clones made for sequence analysis of the *fbp1* gene. A 240 bp HindIII BamH1 fragment containing about 220 bp promoter sequence and 14 bp downstream of the *fbp1* ATG was isolated and together with a 750 bp EcoRI/HindIII fragment isolated from another sequence deletion clone cloned into the Bluescript KS (Stratagene) vector yielding pFBP15. From this subclone a 1090 bp SalI/BamHI frgament was cloned into the binairy GUS plasmid pBI 101.3 (Jefferson et al; 1987) yielding plasmid pFBP12E with a 1090 *fbp1* promoter fused in front of the GUS reporter gene (Figure 2B).

III Construction of Chimeric 35S CaMV-*fbp1* and 35S CaMV-fbp2 genes.

The plasmid pBI121 (Jefferson et al., 1987) was digested with EcoRI and SstI and the resulting nos terminator fragment was cloned into pBIN19 (Bevan et al., 1984) rendering pBINT. Subsequently a pDIP22 (a Bluescript plasmid containing the full size *fbp1* cDNA) XbaI/KpnI fragment was cloned into pBINT cut with XbaI and KpnI. Finally in this subclone a XbaI/BamHI 35S CaMV fragment isolated from the plasmid pCAL1Gc (Plegt et al., 1992) was cloned yielding plasmid pFBP20 (Figure 2C). Furthermore a pDIP63 (a Bluescript plasmid containing the full size *fbp2* cDNA) EcoRV/XbaI

fragment was cloned into pBINT cut with SmaI and XbaI. This yielded a subclone in which a XbaI/BamHI 35S CaMV fragment isolated from the plasmid pCAL1 was cloned yielding plasmid pFBP21 (Figure 2D).

IV Generating Transgenic Petunia Plants.

The binary vector containing the fbp1-GUS construct (pFBP12E) or the 35S CaMV-fbp1 construct (pFBP20) were transferred from E. Coli JM83 (Messing, 1978) to *Agrobacterium tumefaciens* strain 4404 (Hoekema et al., 1983) by triparental mating (Rogers et al., 1986), using a strain containing plasmid pRK2013 (Ditta et al., 1980). Exconjugants were used to transform *Petunia hybrida* leaf discs, as described by Horsch et al (1985). Leaf disks were prepared from top leaves of young, non-flowering *Petunia hybrida* variety W115 plants. After shoot and root induction on kanamycin containing media, plants were put on soil and transferred to the greenhouse. Plants regenerated from leaf discs treated with the LBA 4404 strain lacking a binary vector served as a control.

V Analysis of transgenic plants expressing the GUS construct, containing the 35S CaMV-fbp1 construct or containing the 35S CaMV-fbp2 construct.

To study the organ and cell specificity and the temporal behavior of the *fbp1* promoter during floral development the transgenic petunia plants carrying the fbp1-GUS construct were analyzed for *fbp1* driven GUS expression using the methods described in the Experimental part.

Fourteen independent transgenic Petunia plants were generated and analyzed. From the transgenic plants eight showed a clear GUS expression in fluorigenic assays using petal or stamen tissue extracts (Figure 3). No GUS enzyme activity above the background was measured in other tissue (roots, stem, leaves, pedicel, sepals, carpels) than petals and/or stamens suggesting that the *fbp1* promoter is only active in those two types of tissues.

Histochemical analysis revealed that the *fbp1* promoter is activated at the time that the floral primordia start to differentiate into petal and stamen primordia suggesting that the *fbp1* gene determines the development of petals and stamens.

Seven independent transgenic plants were generated which contained the pFBP20 gene. One of those plants showed phenotypic alterations in the petals. These alterations are the result of the socalled co-suppression effect. The petals of this transgenic plant showed a partial conversion of petal tissue into sepal tissue based of the fact that green sepaloid tissue was formed on top of normal white coloured petal tissue. The green tissue possesses trichomes (hairs) on both sides as in the case of sepals whereas normal petals only have trichomes on the outer side. Furthermore this primary transgenic plant has anthers with the top partially

transformed into carpel tissue: on the top of the anthers a short style and a stigma develops. No morphological alterations were observed in any of the other plant parts. When the primary plants were selfpollinated the offspring could be divided into three classes (21 plants analysed). Class I (6 plants) consisted of plants with a normal W115 phenotype and did not contain the transgenes. Class II plants (10 plants) had a phenotype resembling the primary transformant. A backcross of this plant with W115 resulted in a 1:1 segregation of normal W115 plants and transgenic plants. Class III (5 plants) had severely affected flowers with petals almost completely transformed into sepals and stamens converted into carpels. These carpels were partially or often also completely fused to each other but also to the two whorl 4 carpels. Backcross of these plants with W115 plants resulted in a progeny with 100% primary transformant-like plants indicating that the severely affected plants were homozygous for the introduced genes. When plants with severely affected flowers were pollinated with control W115 pollen, seedset was observed not only in whorl 4 but also in the whorl 3 gynoecium. Therefore it was concluded that a inhibition of *fbp1* expression in transgenic plants could result in extra seedset.

In summary it can be concluded that the *fbp1* gene is a gene specifically involved in the formation of petals and stamens. Furthermore from the GUS data it can be concluded that the *fbp1* promoter retains its specificity in transgenic petunia plants and that using co-suppression techniques petunia floral morphogenesis can be modified using a *fbp1* coding sequence.

Ten independent transgenic plants were raised containing the 35S CaMV-*fbp2* construct (pFBP21). Two of the transgenic plants had modified flower organs as the result of co-suppression inhibition. One plant has flowers with a greenish corolla and petal tissue has been formed on top of the anther. The other plant also exhibited a normal phenotype with the exception of a completely green, short corolla with unfused limbs and a short carpel. From the analysis of these transgenic plants we conclude that the *fbp2* gene has an important role in the formation of petals and stamens. Obviously this cDNA represents a molecular handle to modify flower morphology by reverse genetic methods.

VI Activity of the *fbp1* Gene in Cucumber.

With the use of the particle gun construct pFBP12E containing the *fbp1*-GUS construct was introduced into cucumber petals. Petals bombarded with pFBP12E showed a high number of blue staining cell groups as a result of transient activity of the *fbp1* promoter driving the GUS reporter gene. This indicates that *fbp1* promoter is active in cucumber petals.

The same *fbp1*-GUS construct was also transformed into the genome of cucumber variety Isfahan using *Agrobacterium* mediated transformation procedure starting from hypocotyls (see also under VII). Ten independently transformed cucumber plants were generated, 4 of which were analysed in detail for *fbp1* driven GUS expression. Specific GUS expression was observed in petals and stamens of male flowers and petals of female flowers. This indicates that the *fbp1* promoter retains its specificity in transgenic cucumber plants.

VII Generating Transgenic Cucumber Plants.

Transgenic cucumber plants were generated and tested for GUS activity. The transgenic plants showed a clear 35S CaMV driven GUS activity in the first leaves. Southern blot and PCR analysis revealed that those plants contained one gene copy which was integrated in the proper way in different chromosomal positions. From these transformation experiments it was concluded that the developmental stage of the cucumber seedlings is essential for an efficient transformation and regeneration of cucumber transgenic plants. Figure 4 shows the optimal developmental stage of the seedlings. Using our transformation procedure a transformation efficiency of 3 % was obtained.

VIII Removal of Cucumber Flowers Results in Partial Resistance Against *Frankliniella occidentalis*.

As can be deduced from Figure 5 a reduced number of *Frankliniella occidentalis* insects were present on the leaves of treated plants. This was accompanied by a significantly reduced amount of damage ten weeks after sowing on leaves of cucumber plants from which the flowerbuds were removed (compartments A and C) compared to untreated control plants from compartments B and D (Figure 6). Eighteen weeks after sowing the percentage of damage on the top leaves was also significantly less on leaves of treated plants (Figure 7) wereas the overall damage was comparable. In the course of the experiment it was observed that migration of the insects to higher parts of the plants was delayed in the compartments were the flowerbuds removed from the plants. No effects were observed of the removal of flowerbuds on the number and weight of the cucumber fruits of this parthenocarpic plant species (Figure 8).

Taken together these results indicate that removal of flower parts disturbs the cucumber/*Frankliniella occidentalis* relation and that the treated cucumber plants possess a partial resistance against the pest insect without altering the yield of fruit production.

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CLAIMS.

1. A recombinant polynucleotide which can be suitably used for obtaining a plant with modified flowers having the petals and/or stamens completely or partially removed or transformed into another type of organ essentially comprising:
(a) an inhibitory gene either being a gene capable of inhibiting expression of a target gene in the said plant encoding a MADS box protein involved in flower organogenesis developmental programmes or being a toxin gene causing cell death, and
(b) a promoter that is active in the petals and/or stamens of said plant, operably linked to said inhibitory genes as to achieve expression thereof in floral organ primordia of said plant.

2. A recombinant polynucleotide according to claim 1, characterized in that the target gene encodes a MADS box protein specifying the determination of floral primordia into petal and/or stamen primordia.

3. A recombinant polynucleotide according to claim 2, characterized in that the target gene encodes the *fbp1* or *fbp2* proteins as defined herein or a MADS box protein homologous therewith.

4. A recombinant polynucleotide according to any of claims 1 to 3 wherein the inhibitory gene is an antisense gene, a sense/co-suppression gene or a toxin gene.

5. A recombinant polynucleotide according to any of claims 1 to 4 wherein the promoter comprises a MADS box gene promoter or a fragment thereof, which is specifically and to a high level active in the flower perianth and/or stamen primordia.

6. A recombinant polynucleotide according to claim 5, characterized in that the promoter comprises the *fbp1* promoter.

7. A method for obtaining a plant with altered floral morphology comprising the steps of
(a) transferring a recombinant polynucleotide according to any one of the claims 1-6 to cells of a wild type plant with normal flowers,
(b) generating whole new plants from cells having incorporated said recombinant polynucleotide, and
(c) selecting a plant with altered flower morphology having the petals and/or stamens completely or partially removed or transformed into another type of organ.

8. A method according to claim 7, in which the plant is cucumber (*Cucumis sativis*).

9. A method for obtaining a plant with altered floral morphology according to claim 7 in which a plant is selected with altered flower morphology having the stamens completely or partially transformed into carpels.

10. A recombinant plant genome, comprising incorporated therein a recombinant polynucleotide according to any one of claims 1-6.

11. A plant with altered flower morphology obtainable by the method according to claims 7 to 9.

12. A cell, organ, fruit, seed, or progeny derived from a plant with altered flower morphology according to claim 11.

13. A method to protect a plant against pest insects by altering the flower morphology of the plant by introducing a polynucleotide according to claims 1-6 in the genome of the plant.

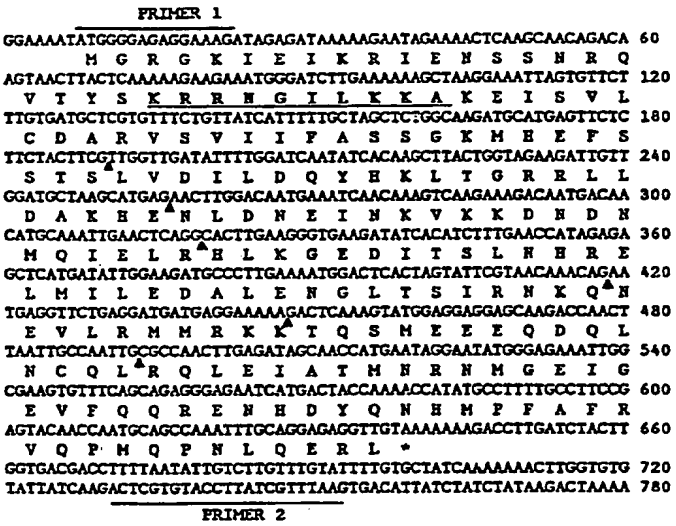
14. A method according to claim 13, in which the plant is cucumber.

15. A method according to claims 13 or 14, in which the pest insect is *Frankliniella occidentalis*.

16. A method of isolating MADS box genes from plant species using a Polymerase Chain Method (PCR) and a primer deduced from a decapeptide amino acid sequence KRRNG(L/I)(L/F)KKA.

17. A method according to claim 16 using the oligonucleotides 5'-GGGGTACCAA(G/A)CGI(C/A)(G/A)I(A/C)(C/A)(T/C)GG(I/C)(T/C/A)T(I/C)(A/T)T(G/C/T)AA(G/A)AA(G/A)GC-3' and 5'-CCGGATCCTCTAGAGCGGCCGC$(T)_{17}$-3'.

A



B

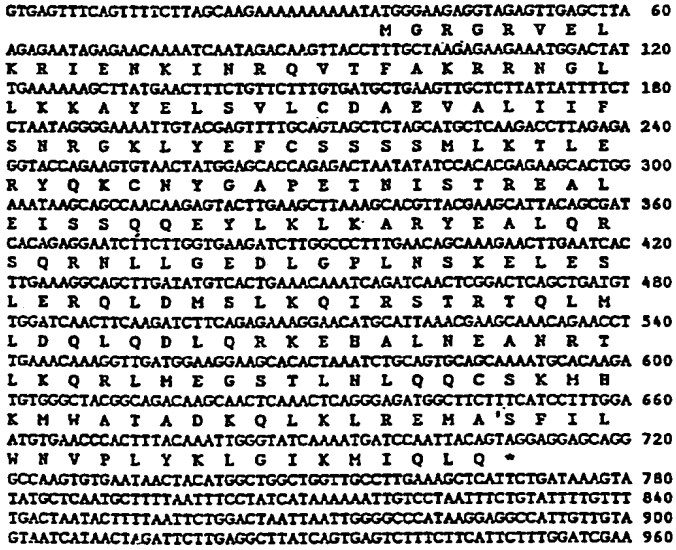
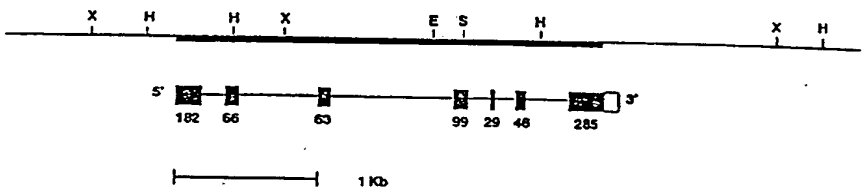
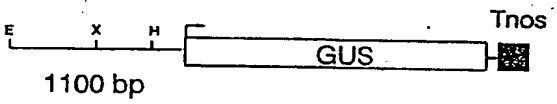


FIG. 1

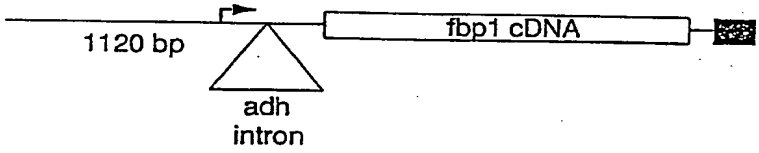
A GENOMIC STRUCTURE fbp1 GENE



B fbp1 PROMOTER - GUS GENE CONSTRUCT



C 35S CaMV PROMOTER - fbp1 cDNA (pFBP20)



D 35S CaMV PROMOTER - fbp2 cDNA (pFBP21)

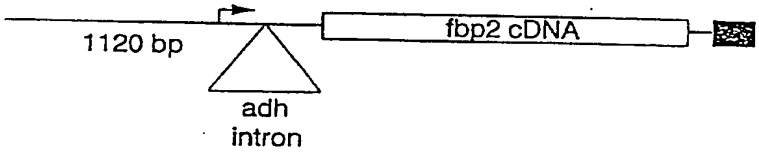


FIG. 2

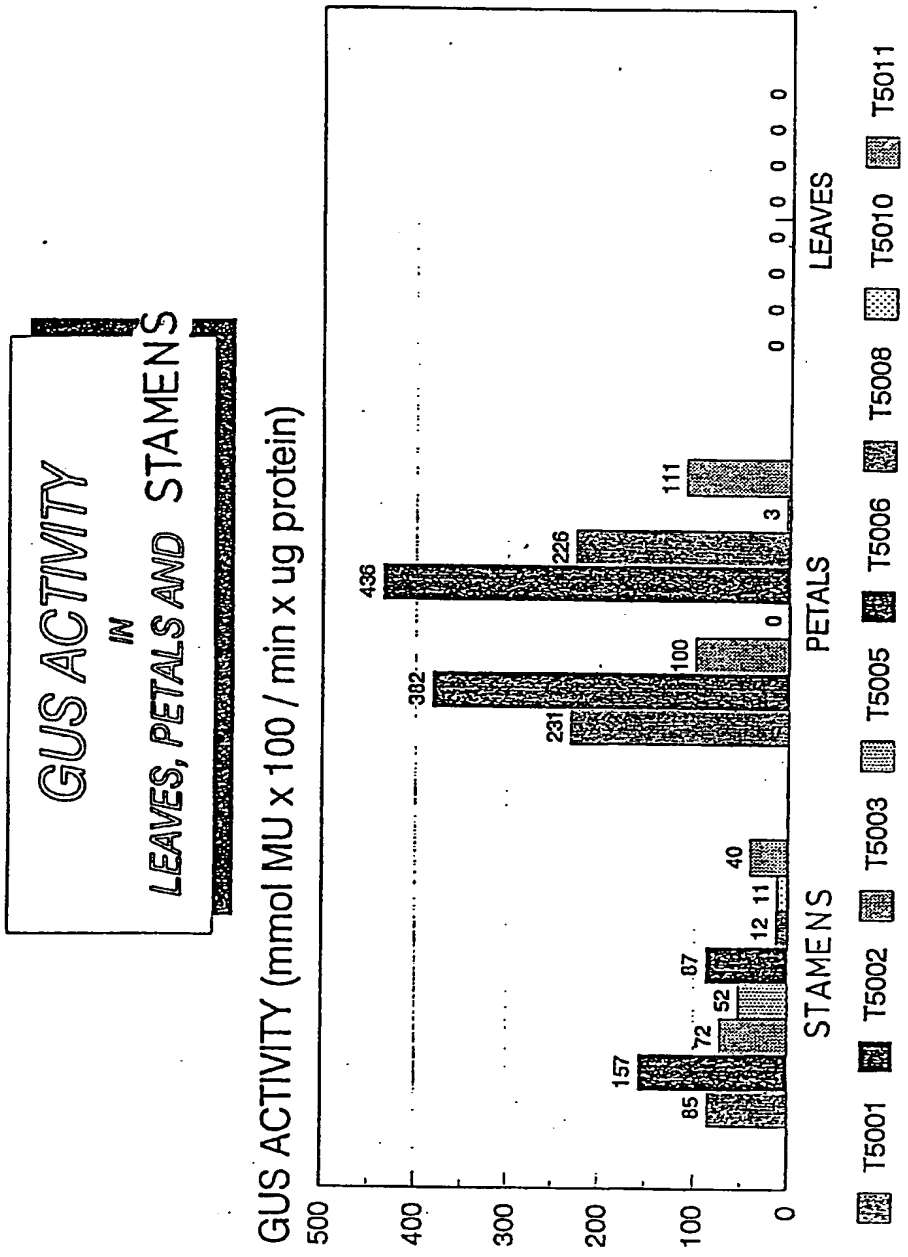

GUS ACTIVITY
IN
LEAVES, PETALS AND STAMENS
GUS ACTIVITY (mmol MU x 100 / min x ug protein)
500
400
300
200
100
0
85
157
72
87
52
12
11
40
231
382
100
0
436
226
3
111
0 0 0 0 0 0 0 0
STAMENS
PETALS
LEAVES
T5001
T5002
T5003
T5005
T5006
T5008
T5010
T5011

FIG. 3

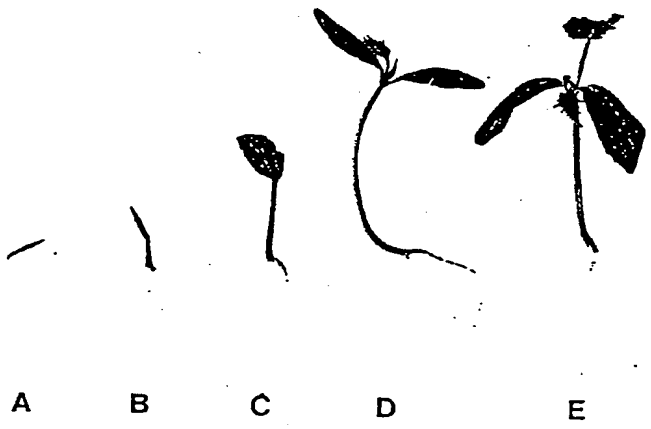


FIG. 4

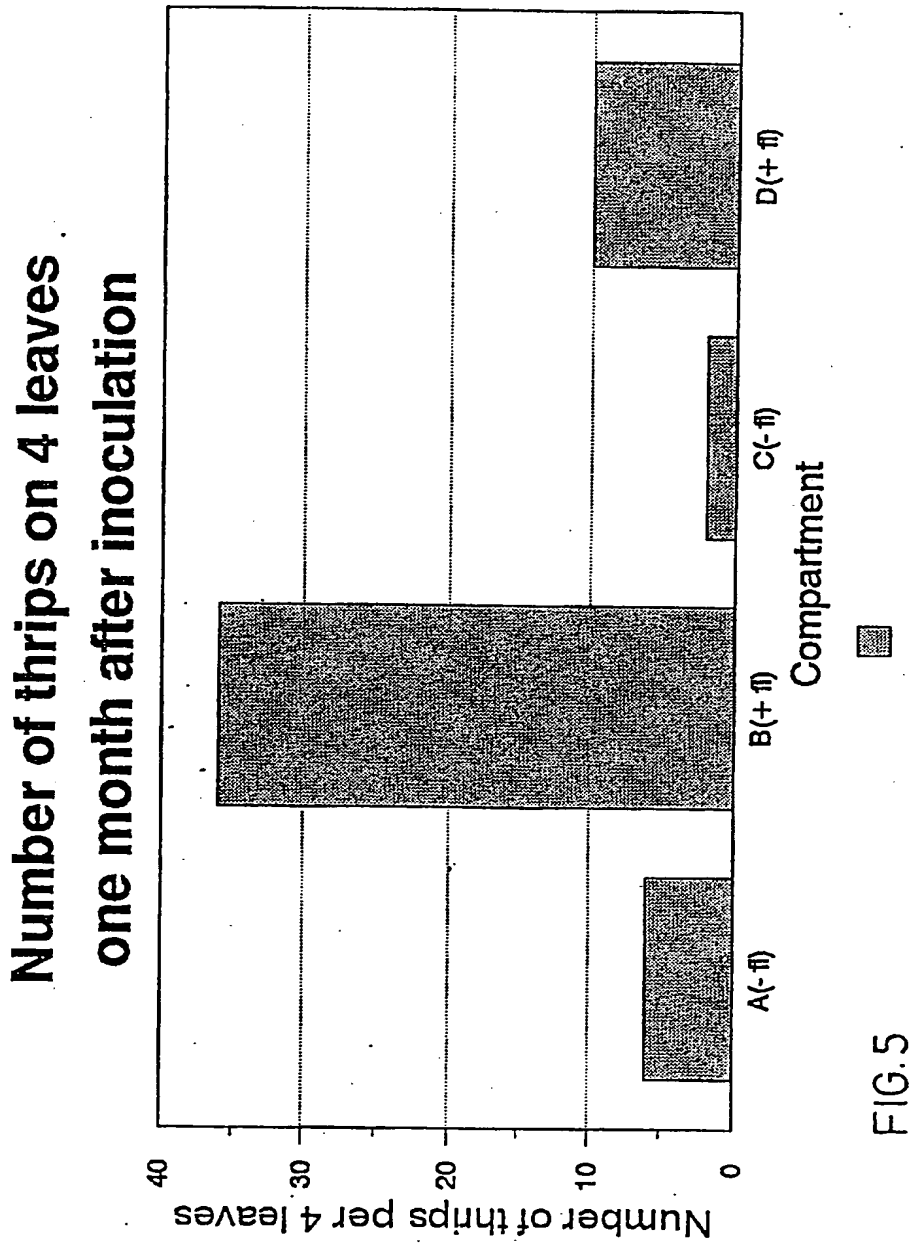

Number of thrips on 4 leaves
one month after inoculation
Number of thrips per 4 leaves
0
10
20
30
40
A(- fl)
B(+ fl)
C(- fl)
D(+ fl)
Compartment

FIG.5

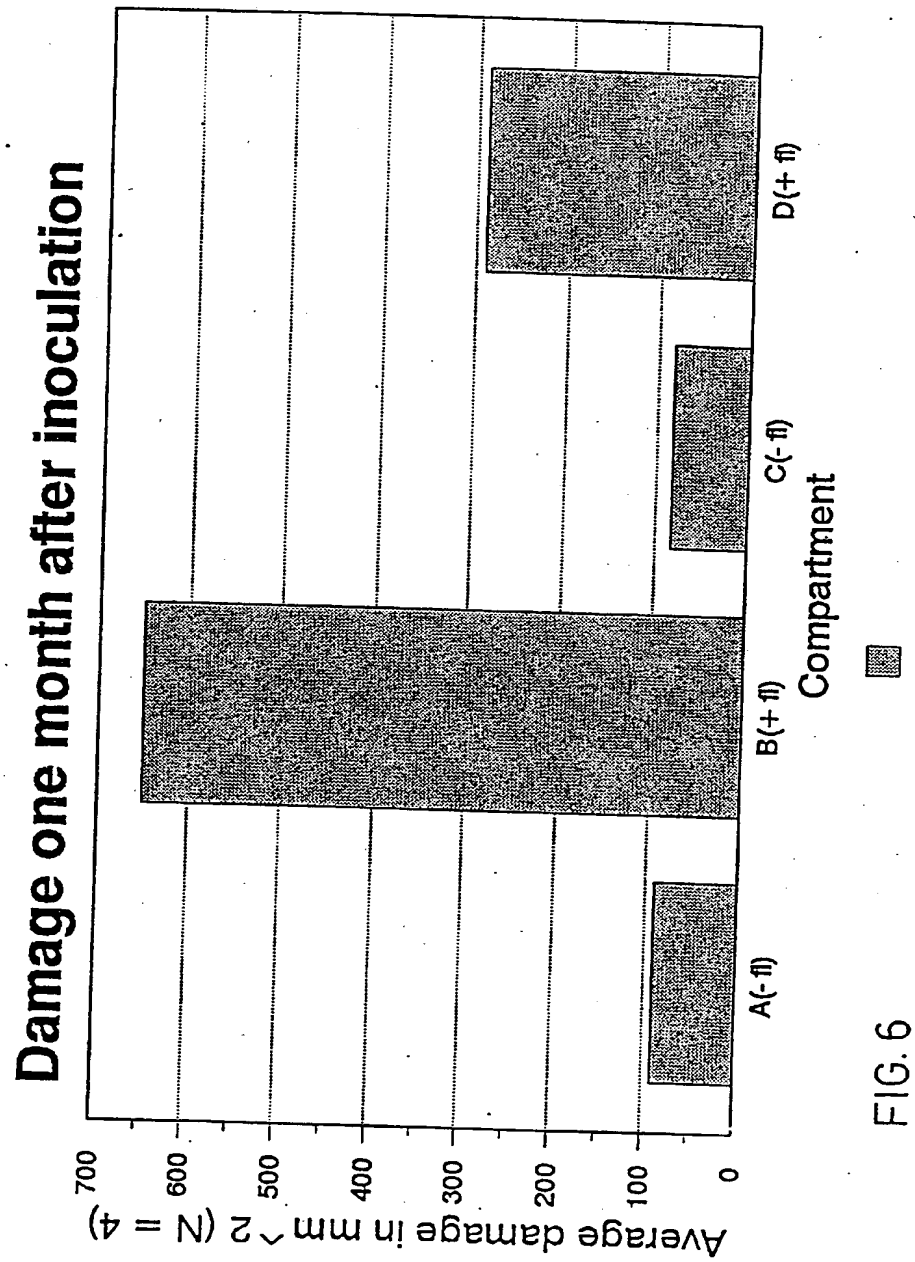

Damage one month after inoculation
Average damage in mm^2 (N = 4)
0
100
200
300
400
500
600
700
A(- fl)
B(+ fl)
C(- fl)
D(+ fl)
Compartment

FIG. 6

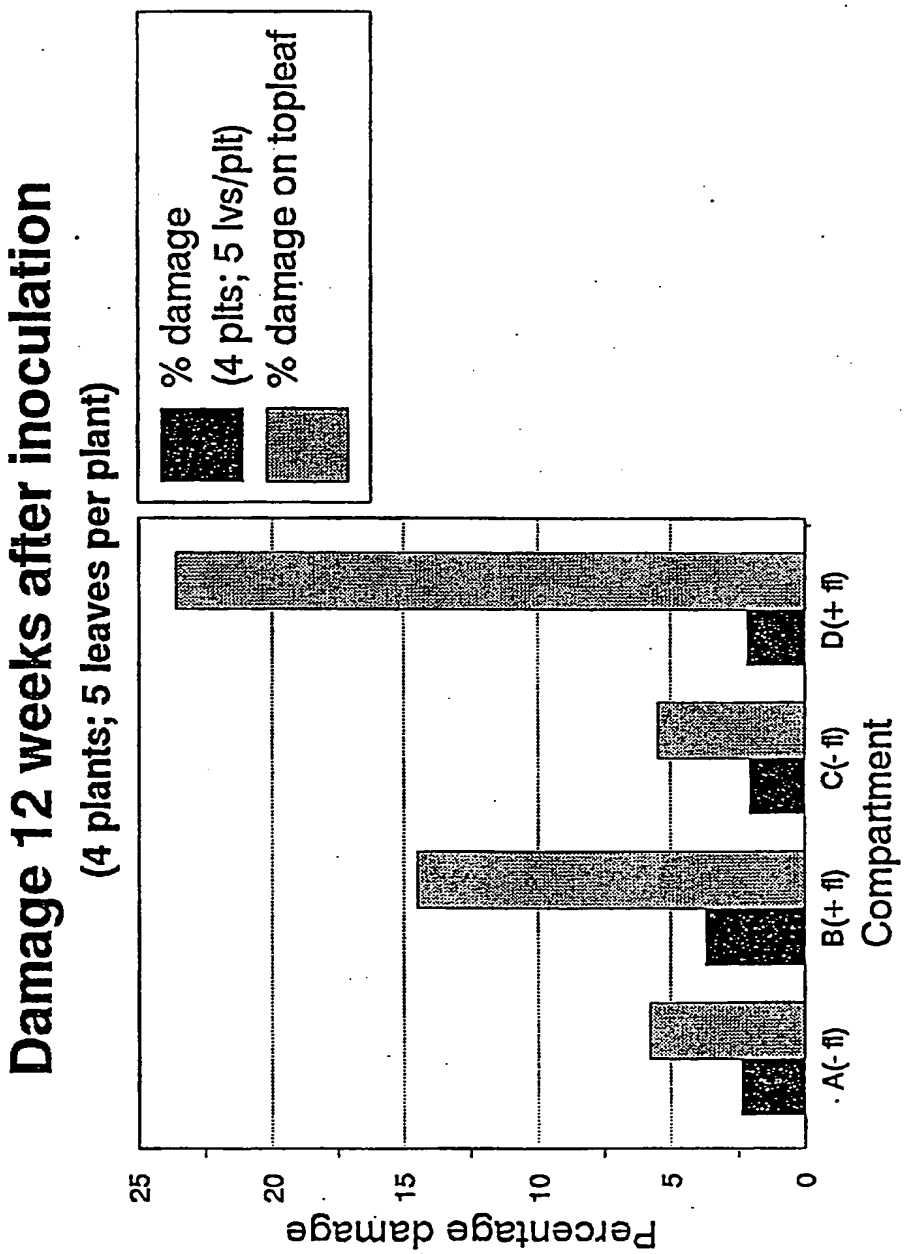

Damage 12 weeks after inoculation
(4 plants; 5 leaves per plant)
% damage
(4 plts; 5 lvs/plt)
% damage on topleaf
Percentage damage
0
5
10
15
20
25
A(- fl)
B(+ fl)
C(- fl)
D(+ fl)
Compartment

FIG. 7

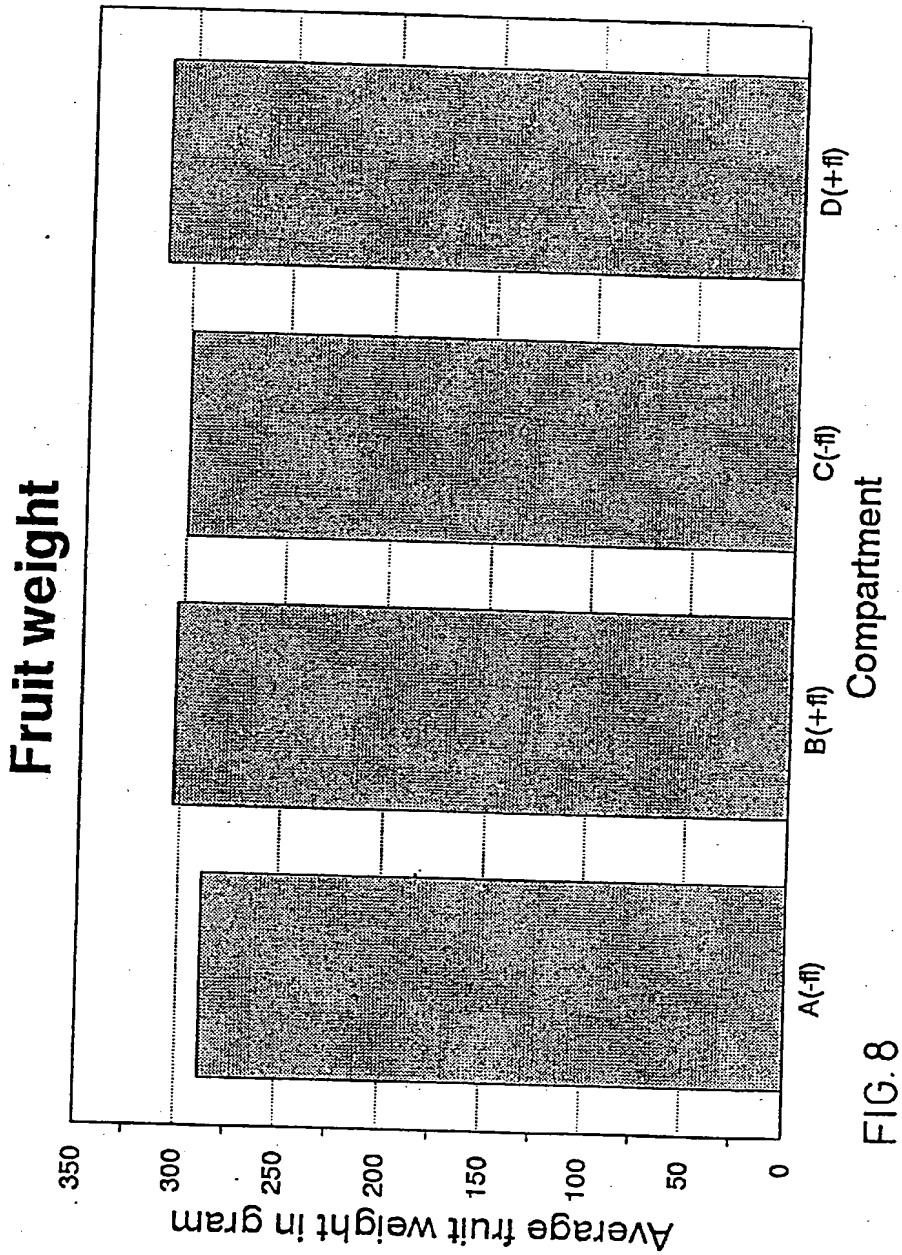

Fruit weight
Average fruit weight in gram
350
300
250
200
150
100
50
0
A(-fl)
B(+fl)
C(-fl)
D(+fl)
Compartment

FIG. 8

FIG. 9

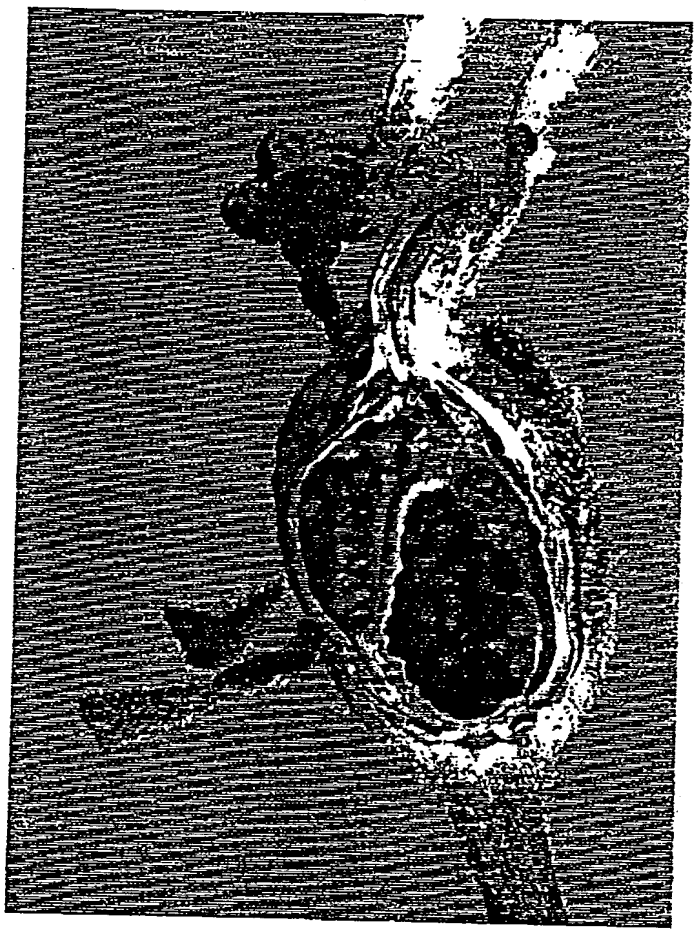


FIG. 10